Downregulated the expression of DNMT3B by suppressing PI3K/Akt signaling pathway and enhances the chemosensitivity of glioblastoma to temozolomide.

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Research Article

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Abstract

Purpose

Glioblastoma is the most common malignant brain tumors and has the poorest prognosis. And a poor prognosis is attributed to chemoresistance to temozolomide (TMZ), the first-line drug for treating glioblastoma. This study aimed to investigate how to enhance the chemosensitivity of glioblastoma to temozolomide.

Methods

Human glioblastoma cell line U251 was used to established temozolomide-resistant U251 (U251-TMZ) cell line by stepwise induction of temozolomide-resistant strains. Reverse Transcription-quantitative PCR (RT-PCR) was applied to detect chemoresistance-related gene expression. Following DNMT3B-siRNA lentiviral vectors transfection and suppressing PI3K/Akt signaling pathway, western Blotting (WB) and RT-PCR were applied to detect DNMT3B gene, p-Akt, t-Akt, p-PI3K, t-PI3K protein expression and cell apoptosis was detect flow cytometry analyses.

Results

Whole-transcriptome analysis revealed that the level of DNMT3B gene expression was significantly up-regulated in U251-TMZ cell line compared to U251 cell line. Moreover, we found that DNMT3B down-expression is correlated with increasing the chemosensitivity of glioblastoma cells to TMZ. Meanwhile, we also found that p-Akt and p-PI3K protein expression in U251-TMZ cells were significant elevated compared with U251 cells. Subsequently, the PI3K/Akt signaling pathway was suppressed using LY294002, leading to a notable inhibition of PI3K phosphorylation and a significant decrease in DNMT3B expression in U251-TMZ cells.

Conclusion

DNMT3B down-expression can inhibit the proliferation of glioblastoma cells and induce the glioblastoma cells apoptosis in vitro. Additionally, the PI3K/Akt signaling pathway plays an important role in the chemosensitivity of glioblastoma cells to temozolomide by regulating the DNMT3B expression.

Introduction

Gliomas, aggressive, infiltrative and malignant tumors, originate from the neuroectoderm and account for approximately 81% of all primary malignant brain tumors[1, 2]. Glioblastoma (GBM), a highly infiltrative and aggressive primary malignancy in human central nervous system, has a median survival of 12-15months and an overall 5-year survival rate of less than 5%[1, 3]. Currently, the standard treatments for
GBM primarily comprise surgical resection combined with postoperative radiotherapy, chemotherapy, and tumor-treating fields (TTF)[4–6]. Although radiation therapy has the potential to eliminate radiosensitive GBM cells, chemotherapy remains a crucial component of treatment following surgical intervention. Temozolomide (TMZ), a DNA-alkylating agent and highly efficacious chemical agent, is commonly employed in the management of primary malignant brain tumors by inducing lethal DNA lesions in fast-dividing tumor cells[7–9]. However, resistance to TMZ represents a significant obstacle to successful GBM treatment, necessitating the development of strategies to overcome this resistance and augment the chemosensitivity of GBM cells to TMZ.

Numerous studies have demonstrated that modifications to DNA methylation are significant contributors to the pathogenesis and progression of various tumors, including GBM[10]. Additionally, aberrant DNA methylation has been linked to the development of chemotherapy-resistant tumor phenotypes [11]. Epigenetic modification, a biological process that modifies gene regulation without altering DNA sequence[12, 13], is well-established as a key factor in tumor-related gene expression, including chromatin remodeling, histone modification, and DNA methylation.[10]. DNA methylation, occurring at the 5th position of Cytosines at CpG site, is catalyzed by DNA methyltransferases (DNMTs)[14, 15]. There are several DNMTs mainly involved in DNA methylation, including DNMT1, DNMT3a and DNMT3B. However, the DNMT3a and DNMT3B catalyze the de novo methylation of DNA [16]. Studies of tumor cell lines and primary tumor tissues provide compelling evidence that aberrant change in DNA methylation patterns is a hallmark of cancer[17]. DNMT3B plays a crucial role in embryonic development and the development of aberrant DNA methylation in carcinogenesis[18]. Inhibited DNMT3B expression can reduce the degree of methylation of tumor suppressor genes by demethylation, thereby significantly decreasing the proliferation and invasion ability of tumor cells[19].

The PI3Ks are regarded as one of important chemoresistance-cause factors in the treatment of cancer. Protein kinase B (AKT) is an important downstream effector of PI3K signaling that can modulates several pathway, including stimulation of cell growth, modulation of cellular metabolism, and inhibition of apoptosis. Notably, the PI3K/AKT pathway plays a pivotal role in multidrug resistance by synergizing the upstream and downstream targets involved in the modulation of cellular metabolism, cell growth, and apoptosis [20]. In a prior study, Mei demonstrated the PI3K/AKT pathway regulates the expression of DNMT3B at the transcriptional and post-transcriptional levels in human hepatocellular carcinoma lines for the first time[21]. The PI3K/AKT pathway participated in the mediation of the DNMT3B expression in some cancer but its role in TMZ resistance in GBM cells is rarely reported.

Therefore, we hypothesized that the overexpression of DNMT3B can make GBM cells resistance to TMZ-induced cell death. Interestingly, PI3K/AKT pathway participated in chemoresistance in the treatment of cancer, and it can also mediate the DNMT3B expression. Thus, we could reasonably postulate that suppressing DNMT3B expression plays a tumor suppressor role in GBM in a PI3K/AKT pathway-dependent manner. Therefore, this study was undertaken to probe whether PI3K/AKT pathway regulates TMZ resistance in GBM cells through DNMT3B-expression and the upstream and downstream
biomolecules. This study may enrich the mechanism of TMZ resistance in GBM cells, and provide promising therapeutic for GBM treatment.

Material and methods

Cell lines and cell culture

Human glioblastoma-derived cancer line, U251 was purchased from the Cell Bank Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cell line was cultured at 37°C under a humidified atmosphere of 5% CO₂ by using Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin (Sigma, St. Louis, Mo, USA).

Stepwise induction of drug-resistant strains

A glioblastoma cell line with acquired resistance to TMZ was developed from the U251 cell line. Initially, we cultured U251 cell line in 6-well plates. Following overnight incubation at 37°C, the medium was replaced with a fresh medium containing varying concentrations of TMZ ranging from 50µM to 1600µM for further induction. TMZ treatment was repeated every 24 h for 5 consecutive days, and cells were exposed to fresh TMZ every 3 day for 3 weeks. Then we selected the surviving colonies and established as TMZ-resistant U251 (U251-TMZ) cell lines by MTT assay.

MTT Assay

The cell viability of human glioblastoma-derived cancer line (U251) was detected using the MTT Cell proliferation and Cytotoxicity Assay Kit that purchased from (MedChem Express, Monmouth Junction, NJ, USA) according to the manufacturer’s instructions. Cell lines (1×10⁴ cells/well) were all cultured in 24-well plates and then transfected with Vector/NR5A2 or Scramble/NR5A2-sh2#. The transfected U251 cells were cultured for 12, 24, 48, and 72 h. After that, the MTT solution was removed and replaced with 150µL 4% dimethyl sulfoxide (DMSO; Sigma). Moreover, the half maximal inhibitory concentration (IC50) was detected to determine the cytotoxicity of TMZ. A microplate reader (Bio-Tek, Instruments, Neufahrn, Germany) was used to measure the absorbance at 490 nm. Experiments were done in triplicate.

RNA Extraction, Reverse Transcription-quantitative PCR

Total RNAs were extracted from the cell lines (U251 and U251-TMZ) using TRIzol regent (Invitrogen; Thermo Fisher Scientific, Inc.) based on the manufacturer’s protocol. cDNA was synthesized by using High-Capacity cDNA Reverse Transcription kit with RNase Inhibitor (Fermentas; Thermo Fisher Scientific, Inc.) and Maxima SYBR green/ROX qPCR Master Mix was used (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. qPCR analysis was performed using the QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The program was performed as follows: denaturation at 95°C for 15 min, followed by 40 cycles of 10s at 95°C for annealing, and 32 s at 60°C for extension. The melting curve started at 95°C for 15 s, followed by 60°C
for 1 min and ended with 15 s at 95°C. The primers were shown in supplemental Table S1. The $2^{-\Delta C_T}$ method was used for the relative quantification of gene expression levels following the quantitative real time polymerase chain reaction experiments.

Table I. Sequences of Primers for RT-PCR

<table>
<thead>
<tr>
<th>Gene (Human)</th>
<th>Forward primer</th>
<th>Reversed primer</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT3B</td>
<td>GAGTCCATTGCTGTGGAAACCG</td>
<td>ATGTCCCTCTTGTGCGCAACCT</td>
<td>305</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AAGGCTGAGAACGGGAAGC</td>
<td>GAGGGATCTCGCTCCTGA</td>
<td>68</td>
</tr>
</tbody>
</table>

**Construction of the DNMT3B RNAi Lentiviral Vectors**

Based on the gene sequence of DNMT3B in Genbank (Gene ID: 1789), primers of DNMT3B siRNA and negative control were designed and synthesized by GenePharma (Shanghai, China). The interference primers for Si-DNMT3B(DNMT3B-siRNA) were as follows: 5′-AACAAGACTGAAGACGCA-3′. The primers for the interference control-siRNA were as follows: 5′- TTCTCCGAACGTGTCACGT − 3′. Then U251-TR cells were transfected with control-siRNA and DNMT3B-siRNA lentiviral vectors respectively.

**Flow cytometry analyses**

For cell apoptosis assay, $2\times10^5$ cells were seeded in 6-well plates and transfected with control-siRNA and DNMT3B-siRNA lentiviral vectors respectively. The cells apoptosis was detected using an Annexin V-fluorescein isothiocyanate (FITC) kit (Sigma, St. Louis, MO, USA). Double staining with FITC-conjugated annexin V and propidium iodide (PI) was performed as follows. The cells and floating cells, washed twice with 4°C PBS and resuspended in binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2 mM KCl), were harvested after 48h transfection. Annexin V was added for 15 min in the dark. Then, cells were washed, centrifuged, and resuspended in binding buffer. Before flow cytometric analysis, PI was added to each sample. Annexin V+/PI-cells were early apoptotic cells. Finally, the cell apoptosis detection was performed by a FACS Cano II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

**Colony formation assay**

Following transfection, $1\times10^3$ U251 cells were plated into 6-well plates and incubated at 37°C for 21 days. A colony was defined as a clump of cells that could be clearly distinguished from another clone after staining. Then, cell colonies were fixed with 10% formaldehyde for 20 min at room temperature and stained at 37°C with 0.1% Coomassie Brilliant Blue R250 for 2 min. Cell colonies were visualized using a camera and counted.

**Western Blotting**

Total protein was extracted from the cell lines (U251 and U251-TR) respectively using RIPA lysis buffer (Beyotime Institute of Biotechnology). And the total protein was quantified using a bicinchoninic acid
assay kit (Beyotime Institute of Biotechnology) and 20µg protein/lane was separated via 10% SDS-PAGE. The separated proteins were subsequently transferred onto polyvinylidene fluoride membranes (Millipore Sigma) and blocked with 5% non-fat milk in TBS-Tween-20 (TBST; 0.1% Tween-20) at room temperature for 1 hour. Then, the membranes were incubated at 4°C overnight with primary antibodies including p-Akt (ab81283, 1:800), t-Akt ((ab8805, 1:1000)), p-PI3K (ab154598, 1:800), t-PI3K ((ab32089, 1:1000) and GAPDH (ab181602, 1:1000). Following the primary antibody incubation, the membranes were rinsed five times with TBST and incubated with the secondary antibodies: Horseradish, peroxidase-conjugated goat anti-rabbit secondary antibody (1:10000; cat. no. ab205718; Abcam) and Horseradish, peroxidase-conjugated goat anti-mouse secondary antibody (1:10000; cat. no. ab205719; Abcam) for 1 hour at room temperature. Protein bands were visualized using an enhanced chemiluminescence (Thermo Fisher Scientific, Inc) reagent and densitometric analysis was performed using Image J version 1.5d software (National Institutes of Health).

**Statistical Analysis**

All data analyses were carried out using SPSS 22.0 (IBM, Armonk, New York, USA). All quantitative data are presented as mean ± SEM. Comparison of data between different groups was performed using independent sample t test. The significant levels of statistical tests was set at *P<0.05 and **P<0.001.

**Results**

**Establishment of U251-TMZ**

We firstly establishment U251-TMZ cell lines via increased concentrations of TMZ. The U251-TMZ and U251 cells were treated with different concentrations (0, 50, 100, 200, 400, 800 and 1600µM) of TMZ for 24h. Results from MTT assays showed that U251-TMZ cell viability was not affected when TMZ concentrations were ≤ 50µM within 24h compared to U251. When the TMZ was increased to 100µM, the U251 cell viability was significant decrease compared to the U251-TMZ. And with increased concentrations of TMZ, both the U251 and U251-TMZ cell viability were decrease. But the U251 cell viability significantly decreased compared to the U251-TR in concentrations (100, 200, 400, 800 and 1600µM) of TMZ (Fig. 1). These results indicated that TMZ-resistant GBM cell line (U251-TMZ) was successfully established.

**Differentially expressed genes**

The entire transcriptome of the U251 and U251-TMZ cell line were sequenced. As shown in Fig. 2, 29498 of mRNA genes expression were not altered, and 1175 of mRNA genes expression were significantly decreased (P<0.05), whereas 526 of mRNA genes expression were significantly up-regulated in U251-TMZ cell line, compared with U251 cell line. Further studies by real-time quantitative PCR and western blot analysis, we found that the level of DNMT3B gene (Fig. 3A) and DNMT3B protein (Fig. 3B) expression were significantly up-regulated in U251-TMZ cell line, compared with U251 cell line.
DNMT3B knockout inhibits the viability, colony formation, and enhance the chemosensitivity of glioblastoma cells with the treatment of TMZ.

To investigate the biological function of DNMT3B in glioblastoma cells, DNMT3B-siRNA lentiviral vectors and control-siRNA were transfected into U251-TMZ cell line. The transfection efficiency was evaluated using RT-qPCR and Western blot analysis. DNMT3B-siRNA lentiviral vectors-transfected cells had significantly downregulated expression levels of DNMT3B compared with control-siRNA-transfected cells (Fig. 4A, 4B).

Following the successful transfection, a MTT assay was used to test the cell viability after downregulating expression of DNMT3B. The results identified that the DNMT3B knockout cells showed significantly less cell viability following 24h of incubation with 400µM of temozolomide compared with control-siRNA-transfected cells (Fig. 5). And to determine whether regulation of chemosensitivity by DNMT3B down-expression, we measured DNMT3B knockout cells apoptosis with the treatment of 400µM temozolomide. Flow cytometry analyses showed that the DNMT3B-siRNA lentiviral vectors-transfected cells apoptotic rates were significantly increased compared with control-siRNA-transfected cells (Fig. 6). Meanwhile, colony-forming assay is one of the effective ways to detect the proliferation ability of single cell. We used the clonogenic assay to measure the cell proliferative capacity and viability with the treatment of temozolomide. The colony formation assay revealed that the number of colonies formed in U251-TMZ cells transfected with the DNMT3B-siRNA lentiviral vectors was significantly decreased compared with control-siRNA-transfected cells, suggesting that downregulated expression of DNMT3B can inhibit the glioblastoma cell proliferation (Fig. 7).

The PI3K/Akt signaling pathway regulate DNMT3B protein expression level, thus regulating the chemosensitivity of glioblastoma cells to TMZ

It’s well known that DNA methylation is a crucial regulator in tumor development[10]. And previous study had demonstrated the PI3K/AKT pathway regulates the expression of DNMT3B in some tumor[21]. We further investigated to measure the expression of PI3K/AKT pathway-related proteins (total PI3K, phosphorylated PI3K, total AKT and phosphorylated AKT) in U251 cells and U251-TMZ, respectively. The results showed that phosphorylated Akt (p-Akt) and PI3K (p-PI3K) protein expression in U251-TMZ cells were significant elevated compared with U251 cells, whereas total Akt (t-Akt) and PI3K (t-PI3K) protein levels were not significantly altered in both cells (Fig. 8).

In order to verify the activated PI3K/Akt signaling pathway on chemoresistance of U251-TMZ cells by regulating the expression of DNMT3B, PI3K/Akt signaling pathway was pharmacologically inhibited with LY294002. The result showed that a specific PI3K inhibitor, LY294002, significantly inhibited PI3K phosphorylation and significantly reduced the DNMT3B expression in U251-TMZ cells (Fig. 9). These results indicated that the PI3K/Akt pathway plays an important role in the chemosensitivity of glioblastoma cells to TMZ by regulating the DNMT3B expression.

Taken all results together, the present study revealed that DNMT3B expression levels were positively regulated by PI3K/Akt signaling pathway in GBM, which indicated that activated PI3K/Akt signaling
pathway may increase the DNMT3B expression. DNA methylation tend to increases with increasing the
DNMT3B expression, where chemosensitivity of U251 to TMZ increases with increasing DNA
methylation.

Discussion

As a malignant tumor, Glioblastoma exhibits a higher rate of recurrence and chemoresistance. Patients
often have poor prognosis, causing a huge burden on families and society. Therefore, there remains an
urgent requirement to identify an effective target for therapy. The purpose of our study was to clarify the
underlying resistance mechanism of DNMT3B in GBM cell and the mechanism by which PI3K/AKT
pathway mediates the DNMT3B expression to influence the chemosensitivity of glioblastoma to TMZ. In
this study, we discovered that DNMT3B mRNA is highly expressed in U251-TMZ cell line, and p-Akt and p-
PI3K protein are highly expressed as well. Then, we altered the protein expression of DNMT3B and
suppress PI3K/AKT pathway to improve the chemosensitivity of U251-TMZ cell line to TMZ. In addition,
research in the last decades has shown the aberrant DNA methylation is important in the promotion of
tumor evolution and chemoresistance[11, 22].

Next, we found that downregulation of DNMT3B inhibits proliferation and promotes chemosensitivity in
U251-TMZ cell line with treatment of TMZ in vitro and PI3K/AKT signaling pathway was inactive as well.
As a core factor of our study, PI3K/AKT pathway plays an important role in regulating the expression of
the downstream gene DNMT3B. Recently, DNMT3B mRNA expression is associated with an increased
cancer aggressiveness[23]. And, previous studies demonstrated that DNMT3B overexpression might be
associated with resistance to chemotherapeutics in acute myeloid leukemia and prostate cancer cells[24,
25]. Interestingly, Mei et al. have, for first time, demonstrated that PI3K/AKT pathway regulates the
expression of DNMT3B in hepatocarcinogenesis[21]. This finding is consistent with the results of the
present study. Importantly, the present study demonstrated that suppress the PI3K/AKT pathway by
LY294002 can downregulate the DNMT3B gene expression, and that low DNMT3B expression is closely
related to chemosensitivity to TMZ in U251-TMZ cell. These data suggested that DNMT3B protein is
important for tumor deterioration and plays a crucial role in chemoresistance to temozolomide of GBM.

DNA methylation is controlled by DNA methyltransferases using methyl donor S-adenosyl methionine[21].
DNMT3B, a de novo methyltransferase, is highly expressed and frequently upregulated in many
malignant tumors[19]. And, previous study demonstrated that gene amplification and protein
overexpression of DNMT3B is associated with decreased sensitivity to decitabine and azacytidine in
pancreas and breast cancer cell lines[26]. Also, DNMT3B depletion in DNMT3B-overexpressing colon
cancer cell lines induced apoptosis and inhibited proliferation[27]. We observed that the degree of
DNMT3B mRNA expression in GBM cells inversely correlated with sensitivity to TMZ treatment in vitro,
although TMZ effectively induced apoptosis with inhibition of DNMT3B expression in our U251-TMZ cell
lines.
Moreover, amid the epigenetic therapies, DNA methyltransferase inhibitors has been used for treating tumors, either single or combine\[28\]. we are curious as to whether the DNMT3B mediate the downstream “some genes” hypermethylation and promote GBM chemoresistance to TMZ. And, can concurrent treatment with DNA methyltransferase inhibitors increase the sensitivity of GBM to TMZ? These puzzling and fascinating aspect will be the next stage for our team to explore and study. Also, we need to evaluate the possible role of DNMT3B as a biomarker for TMZ treatment in GBM.

In summary, our finding demonstrated that DNMT3B acts a tumor resistance factor in GBM with the treatment of TMZ. In addition, PI3K/AKT pathway mediates the expression of DNMT3B mRNA, and it suppressed that can downregulate the DNMT3B expression. Meanwhile, downregulation the DNMT3B expression enhances the chemosensitivity and inhibits malignant development of GBM. Our finding provides a new insight into the molecular pathogenesis of GBM and identify DNMT3B as novel therapeutic candidate targets for GBM.

Declarations

Author Contribution Cheng Wang and Wenwu Kan designed this study. Linhui Gao and Wenwu Kan performed the experiments. Linhui Gao participated in study and data analysis. Wenwu Kan wrote the manuscript. And the rest of authors revised the manuscript.

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Data availability All data generated or analyzed about this study are available from the corresponding author with reasonable request.

Ethics Approval and Consent to Participate Not applicable

Consent for Publication Not applicable

Competing interest The authors declare that they have no potential conflicts of interest in the study.

References


Figures
Figure 1

Establishment and validation of temozolomide-resistant U251 cell lines. *P 0.05 **P 0.001.
Figure 2

Volcano plot for differential gene expression of U251 and U251-TMZ cell line. And $P$ values less than 0.05 were considered as the screening conditions.
Figure 3

The expression levels of DNMT3B by real-time quantitative PCR (RT-qPCR) and Western blot analysis. ** $P \leq 0.001$. 
Figure 4

The expression levels of DNMT3B after DNMT3B-siRNA lentiviral vectors and control-siRNA transfected by real-time quantitative RT-qPCR (A) and Western blot analysis (B). ** $P < 0.001$. 
Figure 5

Knockout of DNMT3B enhance the chemosensitivity of glioblastoma cells to TMZ. *P 0.05 **P 0.001

Figure 6

Flow cytometry analyses showed the cells apoptosis (DNMT3B-siRNA cells, early apoptosis: 6.4%, late apoptosis: 21.4%; Control-siRNA cells, early apoptosis: 2.5%, late apoptosis: 14.5%). *P 0.05
Figure 7

The colony formation assay showed that the colonies formed in U251-TMZ cells transfected with the DNMT3B-siRNA lentiviral vectors was significantly decreased compared with control-siRNA-transfected cells. *P 0.05
The Western blot analysis showed p-Akt and p-PI3K protein expression in U251-TMZ cells were significantly increasing compared with U251 cells. *P 0.05

**Figure 8**

![Western blot images showing p-PI3K, t-PI3K, DNMT3B, and GAPDH protein expression in TMZ-resistant and TMZ-resistant + LY294002 conditions.]

**Figure 9**

The Western blot analysis showed that p-PI3K and DNMT3B protein expression in U251-TMZ cells were significantly increasing compared with U251-TMZ cells added LY294002. *P 0.05